Abstract

ANTI-CANCER ACTIVITY OF CUCURBITACIN IIA

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Cancer is a debilitating disease resulting from uncontrolled proliferation. One major treatment strategy for cancer is the application of chemotherapeutic drugs which kill cancer cells. Cucurbitacins are a new family of plant-derived drugs that are being researched for their potential cytotoxic effects against cancer cells. All cucurbitacins have shared an anti-cancer activity stemming from their inhibitory effects on Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling. Cucurbitacin IIa (Cuc IIa) is the newest compound to be characterized in this family. The purpose of this thesis is to investigate the anti-cancer properties of Cuc IIa. Cuc IIa was used to treat mice that had been injected with Lewis Lung cancer cells. These animal studies show that Cuc IIa had reduced the tumor size without any weight change within the mouse. Furthermore, Cuc IIa displays anti-cancer activity against prostate cancer cell lines, PC3 and Rv1, and lung cancer cell line NCI-H1299. Cuc IIa is shown to induce apoptosis through actin clustering. In addition, Cuc IIa induces apoptosis through the increase in cleaved PARP expression and a reduction in phospho-Histone H3 expression. Cuc IIa induces cell cycle arrest at the G2/M phase of the cell cycle. While other members of the Cucurbitacin family express anti-cancer activity through effects on the JAK2/STAT3 pathway, Cuc IIa does not share this feature. Western blot staining for JAK2 and STAT3 proteins reveal that Cuc IIa exterts no direct effect JAK2/STAT3 phosphorylation. Instead, Cuc IIa inhibits

survivin downstream of JAK2/STAT3 phosphorylation. Therefore, this thesis highlights a unique Cuc compound with the potential to become a powerful anti-cancer agent not working at the level of JAK2/STAT3 activation which is commonly found in other Cucurbitacins. This feature may provide a new mechanistic point of anti-cancer drug action to combat drug resistance.

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Chapter 1: Project Summary

Plants are outstanding sources of medicinal chemicals that can inhibit cancer, a deadly disease yet to be conquered. Cucurbitacin (Cuc) and triterpene derived natural products exhibit anti-cancer potential in addition to their conspicuous anti-bacterial and anti-inflammatory activity. Recently, inhibition of JAK2/STAT3 signaling was shown to underlie the effects of Cucurbitacin family members on inducing cell death in many cancer types. Cells have multiple mechanisms for activating apoptosis including activation of the Caspase cascade and inhibition of JAK/STAT pathway. Cleavage of Poly (ADP-ribose) polymerase or PARP, immediate upstream of DNA breakdown as a result of caspase activation, is known to increase dramatically. However, while an increase of apoptosis was indicated through PARP cleavage, in regards to JAK/STAT there was no indication that Cucurbitacin cytotoxicity stemmed from the inhibition of this pathway.

Here, I hypothesize that Cucurbitacin IIa (or Cuc IIa), the active component purified from the medicinal plant *Hemsleya amabilis* Diels, inhibits cancer cell growth, induces apoptosis, and reduces tumor growth *in vivo*. The project was to test this hypothesis and investigate the molecular mechanisms by which Cuc IIa exerts its anti-cancer effects.

I investigated if Cuc IIa is cytotoxic against cancer cells and if the cytotoxic activity stems from acting upon the actin cytoskeleton. I found that Cuc IIa induced the irreversible clustering of filamentous actin and the increases in G2/M cell populations in cell cycle, indicating the disruption of actin cytoskeleton as the likely mechanism of inducing apoptosis, specifically during mitosis.

To better understand the mechanisms of Cuc IIa's cytotoxicity, I focused on cell culture experiments and protein expression analysis of cell cycle and apoptosis. Prostate cancer cell lines, CWR22Rv-1 and PC3, and the lung cancer cell line H1299, were utilized in this study. I confirmed the Cuc IIa cytotoxicity using the cell viability assay to record a reduction in metabolic activity for cells treated with Cuc IIa. The reduced metabolism is used as an indication of cell death based on the difference of metabolic activity between non-treated and Cuc-treated cells.

I also studied the molecular and cellular target mechanisms used by Cuc IIa by immunofluorescence and western blotting analysis. It has been reported that the cytotoxicity of Cucurbitacins stem from interactions with the cytoskeletal proteins actin and microtubule network. Fixed cells stained with actin and tubulin markers indicated an alteration of filamentous actin but not tubulin, as the main cause in association with apoptotic cells. Additional immunofluorescent studies focused on the expression and distribution of MAPK, Rho A and STAT-3 proteins. These proteins are integral in survival and apoptotic pathways. The expression and localization of these proteins under Cuc IIa treatment have shown indications that apoptosis is induced independent of the JAK/STAT pathway. Finally, the Western blot studies of protein expression from the cell lysates confirmed apoptosis by the increased expression of cleaved PARP and a reduction in phopsho-Histone H3 and survivin.

Chapter 2: Introduction

Cancer is a debilitating disease that arises from dysfunction of many critical cellular checkpoints (Maddika *et al.*, 2007). Two hallmarks of cancer are the uncontrolled proliferation of cells and suppression of apoptosis (Evan and Vousden, 2001; Ghavami *et al.*, 2009; Liu *et al.*, 2011). The uncontrolled proliferation typical of cancer cells is a result of defects in the regulatory pathways (Hanahan & Weinberg, 2000). Cell survival pathways, such as JAK/STAT, are commonly over-expressed in neoplasia contributing to oncogenesis (Benekli *et al.*, 2009; Aggarwal *et al.*, 2006; Glienke *et al.*, 2010). Many cancers are initiated through some type of cellular damage either by UV radiation, or cellular stresses.

Mutations in standard cell regulation systems can induce cancer cells to divide without control. It is the continuous division of tumor cells and their metastatic abilities that causes cancer to be so difficult to treat. Chemotherapy drugs are developed with the inhibition of rapid division of cancer cells in mind and induce apoptosis in cancerous cells (Johnstone *et al.*, 2002). The goal of chemotherapeutics is the induction of apoptosis (Longley and Johnstone, 2005). Chemotherapeutics are designed to target specific pathways within the cell, such as inhibiting JAK2/STAT3 (Cucurbitacin I and Cucurbitacin Q) (Shi *et al.*, 2006; Sun *et al.*, 2005). The inhibition of survival pathways and activation of apoptotic pathways are the main targets for developing treatments for cancer (Evan and Vousden, 2001).

Currently, there is a wide variety of treatment options for different cancer types, with chemotherapy being the most effective. Chemotherapeutics work in several different ways. Many act on the cytoskeletal proteins actin and microtubules to induce apoptosis through the

arrest of mitosis, e.g. taxanes on polymerization of microtubules (Jordan and Wilson, 2004) and Cuc E by disruption of actin (Duncan *et al.*, 1996; Duncan and Duncan, 1997). However, severe drawbacks of common chemotherapeutics are their harsh side effects and non-specific targeting. Due to the detrimental side effects, drugs must be administered in rounds with frequent periods of rest in order for the patient to recuperate. These breaks are necessary but they carry the possibility for the cancers to undergo further mutations and acquire a resistance to the chemotherapy drugs. This high level of resistance for cancers, most notable for solid tumors, demands several highly toxic drugs to be used, again causing severe and painful side effects. Chemotherapy drugs are developed based on the faster proliferation rate for cancer cells as opposed to somatic cells (Johnstone et al., 2002). Although chemotherapeutics are effective treatments for neoplastic tissues they also target other rapidly dividing cells, including the cells lining the gut, inside the mouth and rapidly dividing white blood cells.

The categories of chemotherapeutics fall under three categories: alkylating agents, microtubule targeting, and inducers of apoptosis through anti-survival or pro-apoptotic pathways. Alkylating agents include cisplatin type drugs. Cisplatin eradicates cancer cells by cross-linkng the cytosine and guanine nucleotide bases within the cell's DNA, an action preventing the cell from proper replication for cellular division (Brabee 1993; Brozovic and Osmak 2007). This cross linking induces apoptosis in the cell by arresting the cell in G1/S transition of the cell cycle through activation of checkpoints (Gibb et al., 1997). Microtubule targeting drugs include vincristine, vinblastine, and paclitaxa, or taxoll (Torres & Horwitz 1998; Shi 2008). Paclitaxal, derived from the *Taxus brevifolia* Nutt, blocks polymerization of microtubules essential for mitotic spindle formation, resulting in the cell arrest in G2/M phase of the cell cycle (Bhalla

2003; Lanzi et al., 2001; Mukherjee *et al.*, 2001). By arresting the cell in mitosis, the cell cycle checkpoints are activated inducing the cell to undergo apoptosis.

There are several categories for chemotherapy drugs based on the principles of inducing apoptosis in tumor cells or reducing proliferation (Tennant 2010). These actions are performed by blocking a specific pathway in the cell. One attractive signaling pathway to block is JAK/STAT pathway. This has been one of the most successful pathways to be targeted by chemotherapy drugs (Aggarawal 2006). Another pathway to induce apoptosis is through the Caspase-3/PARP pathway which is activated by cleaved caspase-3 which in turn de-activates PARP (Oliver et al., 1998).

In order to better tailor chemotherapy to target cancer cells and overlook normal tissues, many drugs are selected based on characteristics unique to cancer cells. Such characteristics include over-expressed survival pathways (e.g. JAK/STAT), disabled apoptotic pathways (e.g. PARP), and increased cellular division through disabled cellular checkpoints. The JAK pathway is a key pathway to be targeted because this is a survival pathway that cancer cells use to avoid apoptosis to continue proliferation. In many cancers, the STAT pathway is constitutively active, thus transcribing survival proteins such as survivin which assists in avoiding apoptosis. It is assumed that chemotherapy drugs that target the JAK/STAT pathway prevents the inhibitors of apoptosis from being transcribed, and ceases transcription of the proliferation proteins. The development of chemotherapy drugs has focused on survival and apoptotic pathways such as STAT3 and PARP, respectively. It has been found that one of the most successful target pathways is STAT3. By focusing on inhibiting STAT3, researchers have devised a method to induce apoptosis for cancer specific cells.

Another well documented signaling cascade that is affected in many cancers involves PARP as an inducer of apoptosis. The main protein in this pathway, PARP, is responsible for repairing damaged DNA within the cell (Oliver *et al.*, 1998; Augustin *et al.*, 2003). Through caspase cleavage, PARP can disable critical protein and induces apoptosis. PARP is cleaved by effector caspases which are themselves cleaved by initator caspases (Boulares *et al.*, 1999). Caspase-3, which is responsible for PARP cleavage, is activated by Caspase-9 (Cagnol and Chambard 2009; Ndozangue-Touriguine *et al.*, 2008).

Many anti-mitotic drugs target the cytoskeletal proteins such as microtubules and actin (Shi *et al.*, 2008; Gascoigne and Taylor, 2009). Some of them are plant derived drugs, including paclitaxal and other vinca alkaloids (such as vincristine and vinblastine) (Jordan and Wilson, 2004). These drugs are successful due to their actions on microtubules by inhibiting polymerization dynamics (Bhalla, 2003). It has been shown that anti-mitotics induce apoptosis by triggering activation of caspase cascade and PARP.

For thousands of years, many cultures have used plant derived medicines for common illnesses (Ríos 2009; Pezzuto, 1997). Recently, new chemotherapy drugs have emerged that are derived from natural sources. Lately there has been an increase in the use of naturally derived chemotherapy drugs that are growing in popularity due to their reduced incidence of side effects and the multiple pathways these natural drugs affect (Pezzuto 1997; Wu *et al.*, 2002). Several effective drugs include Paclitaxal (Taxus brevifolia L.), Vincristine (Catharanthus roseus G. Don), podophyllotoxin (Podophyllum peltatum L.), and camptothecin (Camptotheca acuminate Decne) (Pezzute, 1997; Kavallaris 2010). Some of the well known plant-derived chemotherapy drugs are also known to work on the cytoskeleton. For example, vincristine works to

depolymerize tubulin while taxol works to stabilize tubulin and cucurbitacin E disrupts the actin cytoskeleton (Momma 2008; Duncan *et al.*, 1996; Duncan *et al.*, 1997; Shi *et al.*, 2008).

The Cucurbitacin compounds are a group of tetracyclic triterpenoids from the cucurbitane skeleton derived mostly from the cucurbitacae family (Haritunians *et al.*, 2008; Chen *et al.*, 2005). Cucurbitacins are characterized by the tetracyclic cucurbitane nucleus skeleton 9β-methyl-19-nor lanosta-5-ene (Figure 1) with a variety of oxygen substitutions at varying locations (Chen *et al.*, 2005; Tannin-Spitz *et al.*, 2007). Cucurbitacins are a new family of naturally derived drugs found in various types of plants and roots across the planet (Graness *et al.*, 2006). The most recent discoveries have identified at least 19 Cucurbitacins ranging from A to S (Chen *et al.*, 2008) which are each distinct within the family and perform a unique function within the cell. Each Cucurbitacin is characterized based on the cytotoxicity level and the cellular signaling pathway it targets (Chen *et al.*, 2005). Interestingly, the majority of the Cucurbitacins have been shown to possess anti-tumor properties (Chen *et al.*, 2008). It has been discovered that most Cucurbitacins induce apoptosis through the JAK/STAT pathway (Tannin-Spitz *et al.*, 2007; Chen *et al.*, 2005). Cucurbitacin compounds, including Cucurbitacin B, are highly selective for the STAT3 pathway, having little to no effect on other cell survival pathways (Graness *et al.*, 2006; Ríos *et al.*, 2009; Thoennissen *et al.*, 2009). Cucurbitacin I inhibits growth of cancer cells with active STAT-3 (Nefedova *et al.*, 2005).

Cuc IIa (25-acetoxy-23,24-dihydrocucurbitacin F, also called hemslecin A; Figure 2), the active component purified from the medicinal plant *Hemsleya amabilis* Diels, is a member of the Cucurbitacin F family (Chen *et al.*, 2005), which has been shown to polymerize tubulin (Maloney *et al.*, 2008). In recent literature, inhibition of JAK2/STAT3 signaling was shown to underlie the effects of Cuc family members on inducing cell death in many cancer types.

Cuc IIa is a triterpene family component of natural products with different structural modifications from other Cuc derivatives. The plant has been used as a homeopathic treatment for various diseases in China for centuries and has been indicated to display anti-cancer potential (Wu *et al*, 2002). However, the molecular and cellular mechanisms of its anti-cancer activities have not been established.

Cuc IIa has been tested for initial cytotoxic effects against cancer cells within animal models, where it has been shown to be effective at reducing tumor growth. Zhang *et al* (2006) tested efficacy of Cuc IIa treatment in a group of mice injected with H22 liver cancer cells and a group of mice injected with Lewis Lung cancer cells. When Cuc IIa was given to the mice by either oral, peritoneal and intravenous injections, it resulted in consistently reduced tumor size. However, the most efficient method for introduction of Cuc IIa was shown to be through intravenous injection. In our preliminary studies, Cuc IIa suppressed cancer cell growth in a dose dependent manner in cell culture. The focus of this thesis is to better understand the mechanisms by which Cuc IIa exhibits its toxicity toward cancer cells.

Chapter 3: Methodology

Cell Culture

Human cancer cell lines NCI-H1299 (lung cancer), PC3 (metastatic prostate cancer), and CWR22-Rv1 (prostate tumor xenograft derived cells), as well as mouse fibroblast NIH3T3 were maintained at 37°C at 5% CO2 environment. The cells are grown in media consisting of RPMI 1640 with 10% Fetal Bovine Serum, and 1% Penicillin/Streptomycin. Some of the cells used have been transfected. These cells require 0.25% Geneticin to be included in the media. The cells will be split at a 1,000 cell density into 96-well plate for MTT assay. The density will range from 10,000 to 100,000 cells grown in 60 mm culture dishes for transfections and immunofluorescence studies.

Cell Proliferation assay

The cells were plated into 96-well trays at a density of 1,000 cells/well and were grown for two days. The cells were treated with the following drugs at Cuc IIa (1, 10, and 100μg/mL), Cisplatin (8, 16, and 32 μg/mL) and Paclitaxel (1, 5, and 10mM) for 48hrs. Then, the cells were incubated in MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (ACROS). Following 3 hour incubation with MTT, 100% Dimethyl Sulfoxide (DMSO) was applied to lyse the cells and release the formazan purple crystal product. The amount of formazan was read at an absorbance of 562nm by a microplate reader (Synergy HT, Bio-Tek).

Cell Growth Assay

In order to record the amount of cell growth over time, H1299 cells were plated into a 12-well tray at 1,000 cells/well. The H1299 cells were treated with Cuc IIa 1 μg/mL, Cuc IIa 10 μg/mL and one set with no treatment. The cells were measured by counting the number of cells in each well on days 4, 6, and 8. The cells were lifted using 0.25% Trypsin and counted using Countess Hematocytometer (Invitrogen). The amount of cell growth was recorded and then analyzed using Sigma Plot.

Flow Cytometry

H1299 cells were plated into 60 mm culture plates at a density of 50,000 cells/plate and grown for two days. The cells were then treated with Cuc IIa at 10 μg/mL for 6hr and 16hr, respectively. Afterwords, the cells were collected for flow cytometry analysis using FlowScan. Specifically, following the 6hr and 16hr treatment, the cells were lifted off the plate using 0.25% Trypsin (GIBCO) for 5min at 37°C. The cells were collected into a 15ml tube and spun down at 1,000 rpm for 5min in order to pellet the cells. After which, the supernatant was discarded and the cells were re-suspended in 300μl of Phosphate Buffered Saline (PBS) and 700 μl of 100% ethanol, then stored in -20°C. To stain the nuclei, the cells are removed from the freezer and spun down at 1,000 rpm for 5min to pellet the cells then remove the supernatant. The cells are washed with PBS one time followed by centrifuge to remove the supernatant. Then the cells are re-suspended in 500 μl Propidium Iodide staining solution (470 μl Phosphate Buffered Saline, 5μl RNase A, and 25 μl of 1 mg/mL Propidium Iodide). The cells were incubated in the stain for 30min at 37°C followed by 1hr incubation at room temperature making sure to avoid light. The DNA content for PI stained cells was analyzed on a FACScan (BD Biosciences, Palo Alto, CA, USA) at 488nm excitation and emission detected using a 585nm band pass filter. The percentage

of cells in each phase of cell cycle (SubG1, G1, S and G2/M) was determined using a ModFit 3.1 computer program (Varity Software House, Topsham, ME, USA). The results were analyzed by taking the mean and standard error results for each phase.

Immunofluorescent stain for apoptotic proteins

Cells are seeded on coverslips in a 24-well plate for two days. The cells were then treated with Cuc IIa at 1, 10 and 50 μg/mL for two days. The cells were fixed using 4% Paraformaldehyde for 20 minutes followed by two washes with PBS, and then the coverslips will be stored in PBS at 4°C. Following permeabilisation with 0.2% Triton for 15 minutes, the cells were rinsed with PBS. The coverslips were incubated in 100mM glycine for 10 minutes followed by a single wash with PBS. The coverslips were then blocked with 10% BSA for 30 minutes at 37°C. Immediately after blocking, the cells were incubated with primary antibody diluted in 1% BSA for 1 hour at room temperature. The primary antibody will be washed off using 1% Bovine Serum Albumin (Sigma) in PBS three times for five minutes apiece followed by incubation with the appropriate secondary antibody for 1 hour at room temperature. The secondary antibody was washed off with three washes of PBS at five min apiece. After the secondary is washed off, the nuclei of the cells were stained with 2μg/mL Hoechest for 1 minute. The coverslips were mounted on microscope slides using AntiFade medium (Molecular Probe).

The proteins that were stained for indicating apoptosis will be JAK2 (1:100), STAT-3 (1:100), and phsopho-Histone H3 (1:100). The secondary antibodies for the primary antibodies were either Cy3 conjugated goat anti-rabbit mouse IgG or Cy3 conjugated AffiniPure Donkey Anti-Mouse IgG (Jackson ImmunoResearch Laboratories, Inc.).

Western Blot analysis of apoptotic and cell proliferation proteins

Proteins were probed using Western blotting method from cell lysates treated with or without Cuc IIa. The cells were grown to 80% confluence in 60 mm culture dishes after which the cells were treated with Cuc IIa at 1 and 10μg/mL for 24hrs. Following treatments, the cells were lysed. The cells were lifted using RIPA Lysis Buffer (Pepstatin A, Complete tablet of protease inhibitor, and RIPA Buffer – Triton, Sodium Deoxycholate, Sodium Dodecyl Sulfide, Sodium Chloride, Ethylenediamine Tetraacetic Acid, and HEPES). The cells were lysed by passing the collected cells through a 22G needle on a 1cc syringe to break up the cells and release the protein. The lysed cells were incubated on ice for 30 minutes followed by centrifugation at 14,000rpm at 4°C for 30min. The supernatant is transferred to a new tube and the protein concentration was analyzed. The protein concentration of the cells was determined using the BCA assay. The lysates were diluted in water and tested against a standard curve BCA concentration. The lysates were diluted in water and tested against a standard curve of BCA concentrations. The lysates and standards were incubated in the BCA kit working solution (Thermo Scientific) for 30min at 37°C followed by 30min incubation at room temperature to cool the solutions. The protein concentrations were determined by reading at 560nm in a microplate reader (BioTek Synergy).

The lysates were run on an 8-16% Tris-Glycine gel in Tris-Glycine running buffer immediately followed by transferring the gel to Nitrocellulose Membrane at 250mA and 90V for a pre-determined timeframe. The membranes were blocked in 5% Bovine Serum Albumin for phosphorylated antibodies and 5% milk for all other antibodies for 1 hour at room temperature. The membranes were incubated with primary antibody at 4°C overnight. The membranes were washed three times with 0.1% Tween-20 in Tris Buffered Saline (TBST) and incubated on

appropriate secondary antibody for 1 hour at room temperature. The membranes were incubated on ECL detection reagent for 1 minute, then exposed to film and developed. The antibodies were PARP, cleaved PARP, phospho-Histone H3, JAK2, STAT3, phospho-STAT3, caspase-3, cleaved caspase-3, and Histone H3. Band density was measured and analyzed using Quantity One (Bio-Rad laboratories). The densities were analyzed in triplicate and the results were averaged and graphed in a chart (Figure 9-right).

Immunofluorescent light microscopy of cytoskeletal proteins actin and tubulin

The cells on coverslips were incubated with 0.2% Triton to permeabilize the cells, and the cells were washed with PBS. They were incubated with 100mM Glycine at room temperature, followed by rinses with PBS. The cells were then incubated with Rhodamine Phalloidin (1:400) at room temperature for 30 minutes. The cells were stained for tubulin using α-tubulin as the primary antibody, using the same method as previously stated when staining for apoptotic proteins. After the coverslips were rinsed three times with PBS, and incubated with 2μg/mL Hoechest, they were mounted on microscope slides using Anti-Fade medium.

Cucurbitacin IIa Molarity

Cucuribtacin IIa has a formula weight of 562.73 g/mol. The concentrations used in this study were 1μg/mL, 10 μg/mL, and 50 μg/mL. In molar concentrations, this equates to 1.8nM, 18nM, and 89nM, respectively. An effective cancer drug is defined as one which has a strong cytotoxic effect at the lowest concentrations possible. Since I have been able to see Cuc IIa induce cytotoxicity in concentrations of nM, this indicates that Cuc IIa could have the potential to be a very powerful tool to combat cancer.

Chapter 4: Specific Aims

Specific Aim #1: To investigate the cytotoxic action of Cuc IIa on cancer cells.

The purpose of this aim is to discover the pathways Cuc IIa effects on the cancer cells. Two pathways that I focused on were PARP cascade and JAK2/STAT3. The methods utilized to investigate these pathways were western blotting for apoptotic and survival proteins as well as immunofluorescence to determine if the protein of interest relocated inside the cell.

Specific Aim #2: To investigate the effect of Cuc IIa on the actin cytoskeleton.

The purpose of this aim is to determine if Cuc IIa induces apoptosis through actions against the cytoskeleton, specifically the actin cytoskeleton. Cucurbitacins of similar structure to Cuc IIa have shown to induce apoptosis through actions against the actin cytoskeleton. We observed morphological changes of cancer cells as a confirmation of actin cytoskeletal disruption which causes cytotoxicity and cell death inhibiting cell locomotion and cellular division.

Chapter 5: Results

Hypothesis: Cucurbitacin IIa induces cancer cell death through the activation of apoptotic pathways and actin clustering.

Specific Aim #1: To investigate the cytotoxic action of Cuc IIa on cancer cells. *– The experimental results I obtained support the conclusion that Cuc IIa shows induction of apoptosis through activation of caspase cascade and mitotic arrest independent of JAK2/STAT3 signaling pathway.*

Cuc IIa, a triterpenoid, shows anti-cancer potential. Metabolic tests, especially MTT assays, are an important indicator of cell viability after the treatment of Cuc IIa. Since formazan is only produced in live cells, the difference in concentration is a reliable method to detect cell death in Cuc treated cells as compared to non-treated cells (Hatok *et al.*, 2009; Yung 1989; Denizot and Lang 1986). It is with this point in mind that the results of the MTT assay show that Cuc IIa consistently killed cancer cells across H1299 cells, PC3 cells and CWR22-Rv1 cells (Figure 3). The cytotoxic effect of Cuc IIa was compared to the amount of cell viability after treatment with Cisplatin. Cisplatin is an anti-cancer drug that is well known to kill cancer cells. Cuc IIa also was compared to paclitaxel. Comparing Cuc IIa to Cisplatin and Taxol revealed Cuc IIa to have a stronger cytotoxic activity (results not shown).

From the MTT assay we were able to show that Cuc IIa decreases cell viability. However it was not known how Cuc IIa affected cell proliferation over time and how soon Cuc IIa affected the cells. In order to determine the continual effects of Cuc IIa, CWR22-Rv1 cells were split onto a 12-well plate at a starting density of 10,000 cells per well. The cells were treated with Cuc IIa at 1 and 10μg/mL and grown over a period of eight days. Each treatment was counted

using a Countess Hematocytometer (Invitrogen) on days 2, 4 and 8. The media was changed and new treatment was added onto the respective wells on days 3, 5 and 7. The number of cells counted was reflected in a graph showing Cuc IIa had an immediate effect on the cells treated (Figure 4). Cuc IIa at 1μg/mL immediately reduced the amount of cells alive within the first 48hr period of treatment. Cuc IIa 10μg/mL had an even more dramatic effect on the cells showing double the amount of suppressed growth in the first 48hr treatment. By counting the number of cells collected, we recorded a decrease in cell number after 24 hours of treatment, with nearly 50% decrease by 1μg/mL Cuc IIa treatment and 100% decrease by 10μg/mL Cuc IIa treatment. It would appear that Cuc IIa at 10μg/mL prevented the carcinogenic cells from proliferating, supporting that the main mechanism of Cuc IIa effects is through actions on the mitotic phase of the cell cycle. Since cells treated 10 μg/mL Cuc IIa never reached above 10,000 cells (initially plated), these experiments suggest that the cells not only did not divide, but they also underwent cell death.

Previous studies on *Hemsleya amabilis* extract, the active component of Cuc IIa, has shown that Cuc IIa has cytotoxic properties toward cancer cells. However it is unknown what phase in the cell cycle that Cuc IIa has the greatest effect. H1299 cells were grown to 60% confluence when the cells were treated with Cuc IIa at 10μg/mL for 16 hours. After collecting and fixing the cells, the H1299 cells were stained with Propidium Iodide and run through a FlowScan flow cytometer to record the percentage of cells in a given cell cycle phase from a sample of 10,000 cells. The results of the flow cytometer showed that Cuc IIa had resulted in a reduced S-phase as well as an increased G2/M phase. This would indicate that Cuc IIa arrests the cell prior to Mitosis in the G2/M phase.

CWR22Rv1 cells were treated with Cuc IIa 1μg/mL and 50μg/mL, respectively, and stained with anti-phospho-RhoA. RhoA is a protein associated with the actin cytoskeleton (Ridley 2006), and is integral in the completion of cytokinesis (Li *et al.*, 2010; Narumiya et al., 2009). RhoA is also well associated with the cytoskeletal arrangements observed in apoptosis (Chang and Lee 2006; Coleman and Olson 2002; Aznar and Lacal 2001). Due to the close association with actin, we wonder if RhoA was affected by Cuc IIa treatment. Since the aggregation of actin can be one effect of Cuc IIa to induce apoptosis, it is of interest to know if Cuc IIa will cause any change in RhoA. From the immunofluorescent staining performed on Rv1 cells, we were able to determine that phospho-Rho A is clustered in a similar pattern as the actin clustering when treated with Cuc IIa (Figure 6).

Not only is the activation of apoptotic pathways a prime target for cancer therapy, there is also an interest in targeting the deregulation of crucial cellular pathways one of which includes the MAPK signaling pathway. There are many studies showing the MAPK pathway is a critical pathway crucial in a wide variety of cellular processes including proliferation, growth, migration and apoptosis, to name a few (Dhillon *et al.*, 2007; Mebratu and Tesfaigzi 2009). Of the MAPK pathways, p38 MAPK pathway has functions inducing apoptosis. It has been discovered the p38 MAPK pathway is disabled in many cancer cell lines (Coulthard *et al.*, 2009). Researchers have found that the p38 MAPK pathway is activated in cell lines treated with the cancer drugs Vincristine, Vinblastine, Taxol and Cisplatin (Dhillon *et al.*, 2007). It is theorized that apoptosis is induced through the activation of p38 MAPK. We sought to determine if Cuc IIa induced a similar phosphorylation of p38 MAPK. H1299 cells were grown to confluency and treated with Cuc IIa at 1, 10, and 50μg/mL followed by immunofluorescent staining using anti-phospho- p38 MAPK (New England Biolabs). In the immunofluorescent staining we expect to see an

increased staining for the Cuc IIa treated cells to indicate MAPK as an element of inducing apoptosis. In the stained H1299 cells, we are able to see an increase in activated p38 MAPK clustering alongside actin fibers. In Cuc IIa 0μg/mL cells, the stain for phospho-p38 MAPK was generally spread out and uniform throughout the cell. In the Cuc IIa 1μg/mL treated cells, we were able to observe the activated p38 MAPK cluster following F-actin. The clustering of phospho-p38 MAPK was increased as the concentration of Cuc IIa was increased. The cells were also stained for actin using Fluorescin phalloidin (Promega) to cause actin fibers to fluoresce green. The actin staining was used to further confirm the actin clustering properties of Cuc IIa observed in CWR22-Rv1 cells. We also stained the nuclei blue with Hoechest as an image reference that allow nuclear/cytoplasm differentiation.

JAK2 and STAT3 are activated when phosphorylated by the serine/threonine kinases on cell surface receptors. These proteins are a part of the JAK pathway to increase cell survival signals while suppressing apoptotic signals. A majority of Cucurbitacins induce apoptosis by inhibiting the JAK2/STAT3 pathway. So, we performed western blot to determine if we could detect any change in JAK2/STAT3 expression level.

Cell lysates were run onto a nitrocellulose membrane and probed for survival proteins JAK2 and STAT3. The western blot staining for JAK2 and STAT3 along with the phosphorylated counterparts showed no consistent differences between treated and non-treated cells, an indication that Cuc IIa may not exert major effects on the JAK/STAT pathway (Figure 8A). This result was supported by immunofluorescent light microscopy showing no redistribution of the STAT3 proteins in the cells (Figure 8B). When activated, STAT3 is known to redistribute from cytoplasm to the nucleus.

The experimental results described above demonstrated that Cuc IIa reduces cell proliferation in a dose- and time-dependent manner, with the first effects seen within the first 48 hours of treatment. Through immunofluorescent staining of the actin regulatory protein RhoA, we can deduce one target of Cuc IIa to induce cell death was through the alteration of the cytoskeleton. Flow cytometry analysis also confirmed the cells were arrested in the G2/Mitosis phase of the cell cycle. With all of this data, we sought to confirm the changes of apoptotic proteins within the cell by Western blots.

The lysates of H1299, PC3 and Rv1 cells were run on a Tris-Glycine gel then transferred to nitrocellulose membrane and blotted for the apoptotic proteins, e.g. Cleaved PARP and PARP. The caspases are a family of cysteine proteases responsible for the morphological changes exhibited in the apoptotic cell (Cohen 1997). Caspase-9 is activated by activated receptors on the cell surface (Ghavami *et al.*, 2009). Then, caspase-3 is activated through cleavage and proceeds to cleave PARP, deactivating its DNA repair function (Gao *et al.*, 2008; Shen *et al.*, 2004). PARP is an enzyme that recognizes breaks in the DNA and is responsible for repairing these breaks (Boulares *et al.*, 1999; Augustin *et al.*, 2003). The cleaved PARP was dramatically increased in cells treated with 50μg/mL Cuc IIa, while the full length PARP protein showed no change within the expression of all treatments.

We also blotted for inhibitor of apoptosis, Survivin and mitotic proteins, Histone H3 and phospho-Histone H3 (Figure 9). Histones are proteins that are responsible for condensing the DNA into bundles, named nucleosomes within the nucleus forming chromatin (Cheung *et al.*, 2000; Rosetto *et al.*, 2010; Taby *et al.*, 2010). The histone proteins release their confirmation and open the DNA when they are active through the phosphorylation of histone H3. Histones, H3 especially, are altered through phosphorylation as a way to make DNA accessible for cellular

processes such as mitosis, DNA repair, and apoptosis (Sawan and Herceg, 2010; Fullgrabe *et al.*, 2010). We detected that phospho-Histone H3 was greatly reduced in cells treated with 50μg/mL Cuc IIa. Survivin, on the other hand, showed a remarkable reduction in expression when cells were treated with 50μg/mL Cuc IIa.

Specific Aim #2: To investigate the effects of Cuc IIa on the actin cytoskeleton. *– The experimental results I obtained support the conclusion that Cuc IIa induces apoptosis through alterations in cytoskeleton by clustering of actin molecules but not against the microtubule network.*

Our studies showed that Cuc IIa have cytotoxic effects on cells through a reduced cell proliferation in MTT assays. To understand how cytoskeletal alterations may affect cell proliferation, CWR22-Rv1 cells transfected with green fluorescent protein tagged actin were treated with 10μg/mL Cuc IIa and recorded every fifteen minutes under the inverted fluorescent microscope for four hours. We observed a drastic increase in actin clustering for cells treated with Cuc IIa as compared to the cells under no treatment (Figure 10). The cells with no treatment continued to grow without showing any signs of a change in cell morphology. However, the Cuc IIa treated cells showed actin clustering over the four hour period as well as a change in the cell morphology. As the treated cells were observed, we detected the cell shrinking and seeming to fold in on itself. The effects on actin with Cuc IIa were reminiscent of that of Cucurbitacin B and E which also affect the cytoskeleton when treating cells in a similar manner.

The very same treatment was performed for the NIH 3T3 cells (Figure 11). These cells were set up on the inverted fluorescent microscope and were also treated with Cuc IIa for a four hour period to record a picture every fifteen minutes. We observed similar results with the 3T3 cells as that with the CWR22-Rv1: cell shrinkage and drastic actin clustering. These results suggested that clustering of the actin fibers is likely one mechanism that Cuc IIa harnesses for the induction of apoptosis.

Since Cuc IIa induced actin clustering in the cell, we sought out to determine if the effect could be reversed. To do this, NIH 3T3 cells were grown and set up under the inverted fluorescent microscope to record a picture every fifteen minutes. NIH 3T3 cells were treated with 50μg/mL Cuc IIa for two hours, after which Cuc IIa was removed. The cells were washed and recorded for two more hours under normal growth media. The actin continued to show clustering under the microscope even after 2 hours following the removal of Cuc IIa (Figure 12).

Many members of the cucurbitacin family have been shown to affect the cytoskeleton. Through our experiments we have shown that Cuc IIa has an effect on the actin, however we don't know if Cuc IIa would also affect the tubulin portion of the cytoskeleton. We grew CWR22-Rv1 cells on coverslips with RPMI 1640 growth media and treated with 50μg/mL Cuc IIa for 24 hours. After 24 hours, the cells were fixed in paraformaldehyde and stained with anti-α-tubulin to label microtubules and Rhodamine Phalloidin for filamentous actin. We observed actin clustering, just as we discovered during the time lapse experiments (Figure 13). However, Cuc IIa did not appear to show any discernible effects on microtubules when they were examined by anti-tubulin staining (Figure 13B). While Cuc IIa has a dramatic effect on actin in the cell (Figure 13A), there appears to be no effect on the tubulin in the cell. The lack of tubulin effects shows Cuc IIa to be different from other cucurbitacins, many of which effect the tubulin, as well as the actin in the cytoskeleton.

Chapter 6: Discussion

Cuc IIa is the most recent compound to be discovered from the Cucurbitacin family, therefore it is expected to have similar apoptotic qualities as the Cucurbitacin family, such as the effects on JAK/STAT pathways. It has been suggested that the inhibition of JAK2/STAT3 underlies the apoptosis when cancer cells are treated with Cuc. Sun *et al* (2005) showed that Cuc effectively inhibited cancer cell expansion only in STAT3-expressing cells. While our study is consistent with the observations of Cuc effects on mitosis, we found that even though prostate cancer cells (CWR22Rv-1) and lung cancer cells (NCI-H1299) displayed JAK2/STAT3 expression and activating phosphorylation, Cuc IIa still induced apoptosis without clearly inhibiting JAK2/STAT3 phosphorylation. Cuc IIa also did not alter nuclear/cytoplasmic distribution of STAT3, as shown by Figure 8. We showed that the cell cycle was disrupted with reduced phospho-Histone H3 and survivin.

Our studies show that Cuc IIa differs from other Cucurbitacins in several interesting ways. As with other Cucurbitacins, Cuc IIa was found to suppress cancer cell growth based on cellular metabolism (Figure 3) and cell growth study (Figure 4). It also disrupts the actin cytoskeleton by inducing non-reversible clustering of F-actin (Figure 12). Whereas other members of the Cucurbitacin family have an effect on JAK2/STAT3 (Haritunians *et al.*, 2008), Cuc IIa does not share this feature, instead induction of apoptosis involves Survivin and PARP, a cascade commonly targeted in chemotherapy drugs. The expression of JAK2/STAT3 in treated cells remained evident compared to that of non-treated cells (Figure 8). In regards to the cytoskeletal changes of Cuc IIa treated cells, we detected a reduction in phosphorylated RhoA. RhoA is a

GTPase protein that is known to regulate the cytoskeleton among other functions (Aznar and Lacal, 2001; Ridley, 2006).

Natural occurring medicinal products have made crucial contributions to anti-cancer therapies. For example Paclitaxel is a taxane-based chemotherapy drug targeting mitotic microtubule cytoskeletons (Pezzuto, 1997). Paclitaxel is originally isolated from the plant *Taxus brevifolia* Nutt acting on cancer cells by stabilizing microtubule cytoskeleton so that the filaments are not able to breakdown properly (Torres and Horwitz, 1998; Cassinelli *et al.*, 2004). As the cell moves through mitosis, the cell is arrested at anaphase (Lanzi *et al.*, 2001). The arrested cell most often goes through apoptosis due to mitotic failure; however, some cells can complete mitosis and then undergo programmed cell death (Shi *et al.*, 2008). While many anti-cancer drugs are designed to alter a single pathway or function, plant-derived drugs have been shown to induce apoptosis in various ways. Also many plant derived drugs have shown to have a direct effect on cell death through the cytoskeletal proteins, namely microtubules and actin.

In recent years, there has been an increased interest in plant-derived chemotherapy drugs due to the successes of the chemotherapeutics Taxol, Vincristine, and Vinblastine. Vincristine and Vinblastine are two drugs derived from *Catharanthus roseus*. These drugs have been successful in treating a large variety of cancers. Also, plant derived chemotherapy drugs target multiple pathways and therefore appear to have a stronger cytotoxic effect on carcinogenic cells. Many plant derived chemotherapy drugs have an added effect due to the pathways involved resulting in apoptosis.

Recently discovered, cucurbitacins are a new class of drugs that are found in plants and roots throughout the planet. Many cucurbitacins belong to a triterpenoid class of drugs that's divided

into 26 categories ranging from Cuc A to Cuc S (Chen *et al.*, 2008). Researchers have found many of the cucurbitacins have cytotoxic properties geared toward cancer cells (e.g. Cuc B). Cuc family disrupts actin dynamics, probably by initiating two signaling cascade events in cancer cells. First, it can inhibit JAK2/STAT3 phosphorylation thereby preventing cell proliferation. Second, the failed cytokinesis activates apoptosis pathways, with reduced phosphorylation level of Histone H3 and increased chromatin damage.

Compounds in the Cucurbitacin family are differentiated based on the chemical structure of the compound and it's affect against cancer cells. There have been many studies investigating a correlation between the chemical structure and the compounds anti-cancer activity. It has been discovered that even one variation on a group off the side chain can indicate a Cucurbitacins activity. Through chemical structure studies, Sun et al. (2005) have identified the side chains on carbon-11 and carbon-3 to be related to the JAK2 and STAT3 of cucurbitacins. Through this study, Sun et al. found cucurbitacins lost anti-STAT3 activity due to the addition of a single hydroxyl group to carbon 11. Sun et al. also found that cucurbitacins lost anti-JAK2 activity when the carbonyl group on carbon 3 was converted to a hydroxyl group. Duncan et al. (1996) also performed chemical structure studies on various cucurbitacins to identify structural differences between cucurbitacins with anti-proliferative and actin-directed cytotoxicity, such as Cuc I and Cuc D. In their studies they found the hydroxylation of carbon 23 along the cucurbitane skeleton eliminated the anti-proliferative and actin disrupting activity typical of many cucurbitacins.

The actin cytoskeleton has a multitude of functions within the cells including cytokinesis, locomotion, and apoptosis (Symons & Mitchison 1991, Kavallaris 2010). Actin is a structural molecule consisting of a globular, ATP binding protein that undergoes polymerization and de-

polymerization in order to grow and shrink as filaments (Staiger & Blanchoin 2006; Watanabe, 2010; Suarez-Huerta *et al.*, 2000). Actin is also a motile protein and essential for growth control and metastasis of tumor cells (Rao et al. 1999). After the cell completes mitosis, cytokinesis occurs and new daughter cell results. When actin is inhibited then the cell becomes arrested during the G2/M phase of the cell cycle thereby preventing cytokinesis. When the cell is unable to divide it is directed into apoptosis. (Brangwynne 2008, Sánchez-Alcázar et al. 2007)

A second cytoskeletal component, which is crucial to mitosis, is the microtubule network. Microtubules have multiple roles including cellular functions but especially important for chromatid alignment and separation during mitosis. Microtubules are mobile as a result of alternating periods of elongation and shrinkage, known as dynamic instability. It has been shown that many Cucurbitacins have an effect on the microtubules leading the cell to undergo apoptosis. Cuc E, for example, stabilizes the microtubules preventing their shortening which draws chromatids back to the daughter cells. The cell becomes arrested in mitosis and undergoes apoptosis as a result of the failed mitotic phase. Through the studies we performed in the lab, it was clear that Cuc IIa had no effect on the microtubules. From the immunofluorescent images where we probed for tubulin in Cuc IIa treated cells (Figure 13B), we were unable to detect any difference between the control and treated cells.

The expression of survivin has two roles in the cell: inhibition of apoptosis and regulation of mitosis. Survivin inhibits apoptosis through interference with caspase-9 preventing the cascade which leads to PARP cleavage and results in apoptosis due to unrepaired DNA damage (Li *et al.*, 1998; Andersen *et al.*, 2007). Survivin is required for the proper completion of cell division during mitosis, partly by ensuring the microtubules are properly attached to kinetochores (Anderson *et al.*, 2007). Many cancers have increased survivin expression as compared to

normal tissues. The increased survivin expression helps cancer cells to prevent apoptosis (Olie *et al.,* 2000). To better understand the cytotoxic effect that Cuc IIa exerts on the cancer cells, we investigated survivin expression in cell lysates. We observed a dose dependent decrease in survivin expression level across the cell lines probed. The downregulation of survivin suggested that the ability of cancer cells to prevent apoptosis during cell cycle arrest is being compromised. Therefore, due to the lack of survivin, the caspase cascade is initiated leading to apoptotic cell death.

Overexpression of survivin and RhoA inhibition in CWR22Rv-1 cells protected cancer cells from Cuc IIa to some extent, suggesting that mitotic-related pathways involving survivin are potential targets to enhance Cuc IIa's anti-cancer potential (which could be cooperating with p53 and p21 pathways). Therefore, not all Cuc family members induce apoptosis by directly inhibiting JAK2/STAT3 phosphorylation. Rather, they could target the converging downstream elements such as survivin, indicating a broader benefit of applying Cuc derivatives. Thus, we hypothesize that Cuc IIa arrests cell cycle progression and results in cell death in the following working model (figure 14).

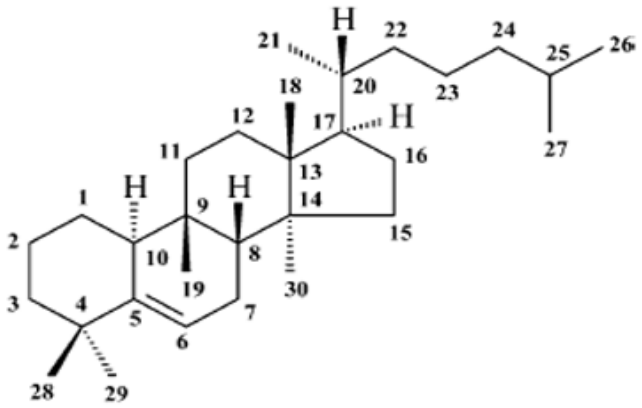
The present study focused on Cuc IIa purified from the Chinese medicinal plant *H. amabilis* and demonstrated, for the first time, that Cuc IIa disrupted actin cytoskeleton and induced apoptosis through novel pathways involving survivin and PARP but independent of JAK2/STAT3 phosphorylation and distribution. Future studies include investigate further the mechanisms by which Cuc IIa exerts its apoptotic functions toward cancerous cells. Mechanisms by which chemotherapeutics target include inhibiting survival pathways and activating apoptotic pathways. One survival pathway of interest is the MAPK pathway. It has been noted that the activation of p38 MAPK leads to apoptosis through the activation of the cell-

cycle regulators p53 and p73 (Olson and Hallahan, 2004). Since Cuc IIa induces apoptosis through downregulating survivin and PARP, it would be of interest to determine if Cuc IIa also induced apoptosis through p38 MAPK downstream activators.

A new direction that can be pursued in regards to cytotoxicity of Cuc IIa is to investigate any association Cuc IIa may have with peripheral neuropathy. Our lab has performed studies on peripheral neuropathy caused by Cisplatin and methods of preventing peripheral neuropathy. Since Cuc IIa is a new drug there is little information on the effects of Cuc treatment on neurons. Testing for any possible neuronal damage could lead to a better understanding of the mechanisms by which Cuc IIa induces cytotoxicity.

Another possible study to pursue is to determine if Cuc IIa cytotoxicity specifically targets cancer cells or if cytotoxicity is non-specific. All of the cells used were cancerous and non-cancerous cells were not applied in this study. To further our understanding of Cuc IIa, we could compare the Cuc IIa treatment of cancer cells to non-cancer cells.

Chapter 7: Figures



Fiure 1: Cucurbitacin basic structure, 9β-methyl-19-nor lanosta-5-ene, shared by all members of the cucurbitaceae family with differences being oxygen substitutions.(Chen et al., 2005)

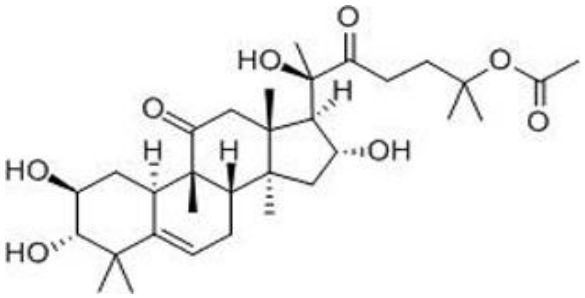


Figure 2: Structure of Cucurbitacin IIa (hesmlecin A)

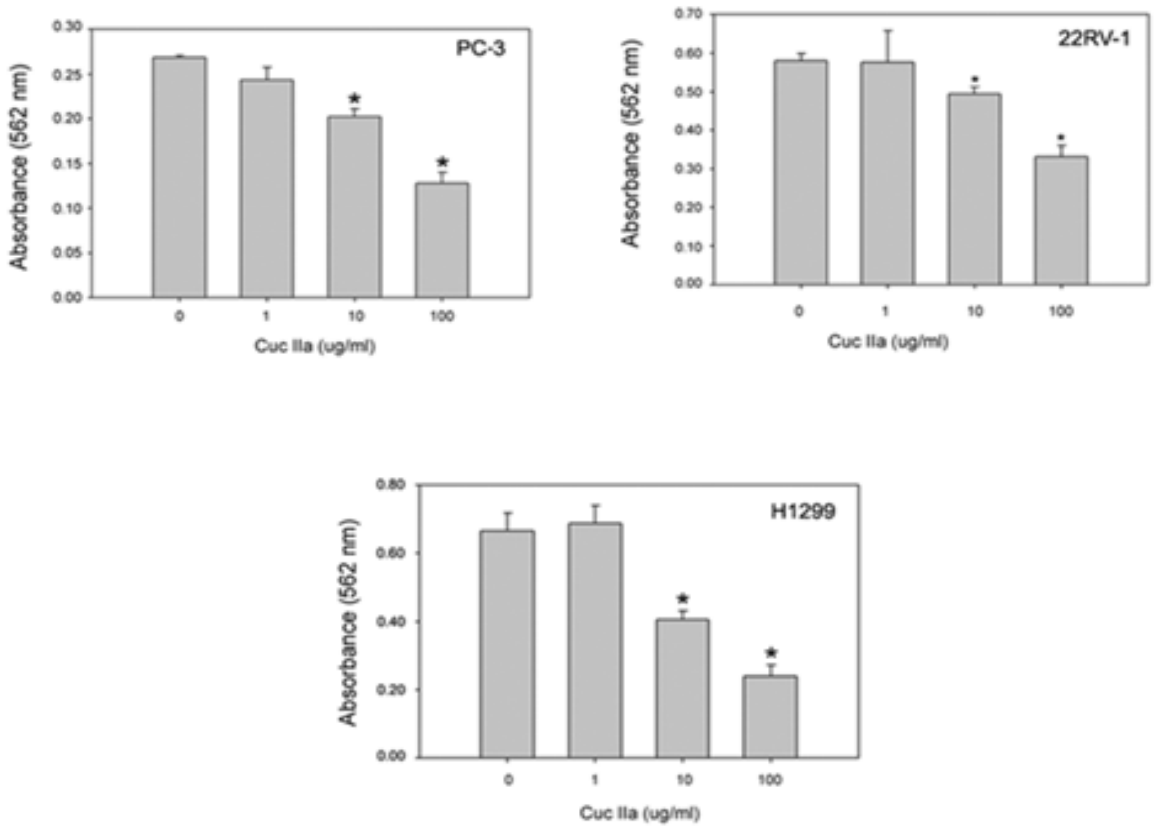


Figure 3: H1299, CWR22Rv-1 and PC3 cells treated in Cuc IIa 1, 10 and 100 µg/mL were observed to have an increased toxicity across the cell lines. Cells were treated with Cuc IIa along with Taxol and Cisplatin we observed Cuc IIa had a consistent toxicity in carcinogenic cell lines. *$P < 0.05$

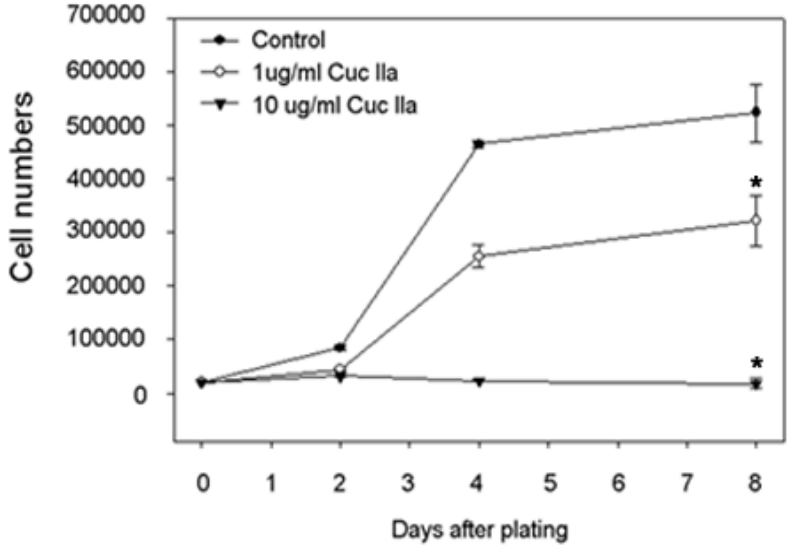


Figure 4: CWR22Rv-1 cells were plated at 10,000 cells/well and allowed to grow for a total of 8 days. Cells were treated on the 1st, 3rd and 5th days with Cuc IIa 0μg/mL, 1μg/mL and 10μg/mL to be collected on the 2nd, 4th and 8th days. *$p < 0.01$.

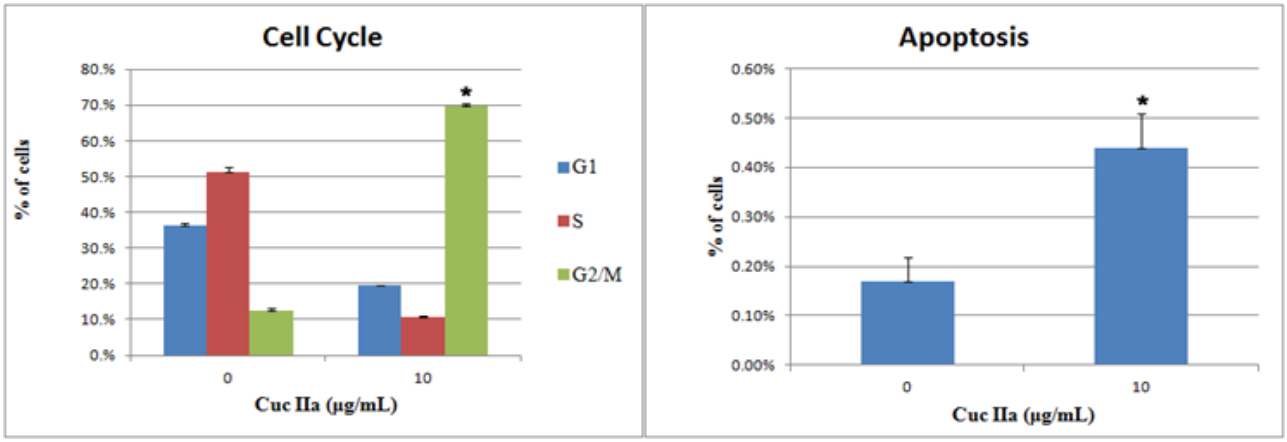


Figure 5: H1299 cells treated with 10 µg/mL Cuc IIa, A) Cell phases G1, S and G2/M; B) Cell phase sub-G1 (apoptosis). Cells were stained with Propidium Iodide and run through flow cytometer to record the phases of Cuc IIa treated cells and non-treated cells. We recorded a majority of cells arresting within the mitotic phase of the cell cycle. Along with the increase in apoptosis for Cuc IIa treated cells, we are able to conclude that Cuc IIa treated cells are arrested within the mitotic phase of the cell cycle. *$p < 0.01$

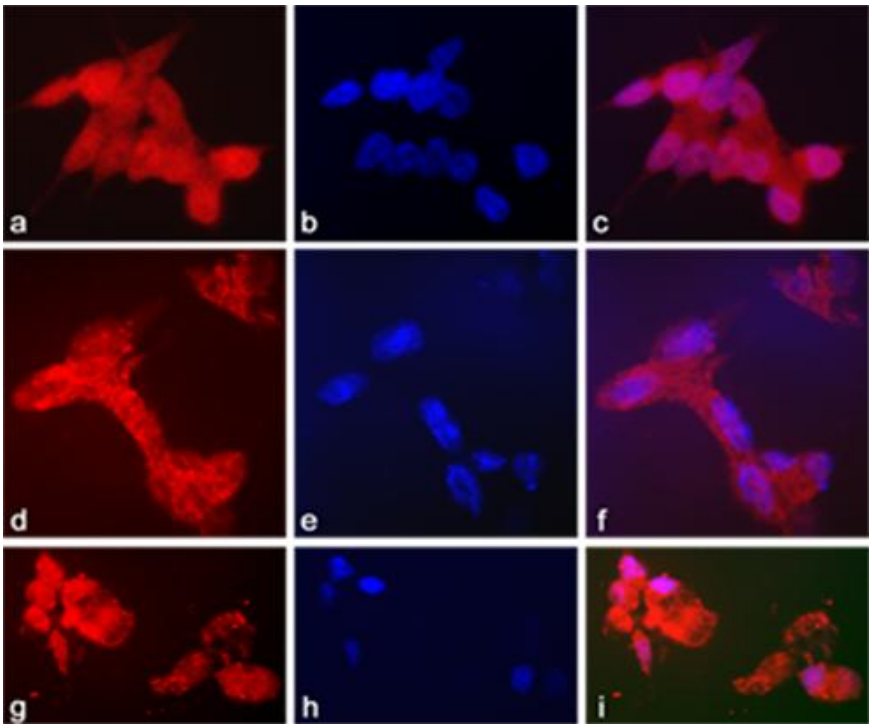


Figure 6: CWR22Rv-1 cells were stained with anti-phospho-RhoA (red) and Hoechst staining to indicate nuclei (blue). a-c: 0µg/mL Cuc IIa, d-f: 1µg/mL Cuc IIa, g-i: 50µg/mL Cuc IIa. a,d, g: phospho-RhoA, b, e, h: nuclei; c, f, i: merge.

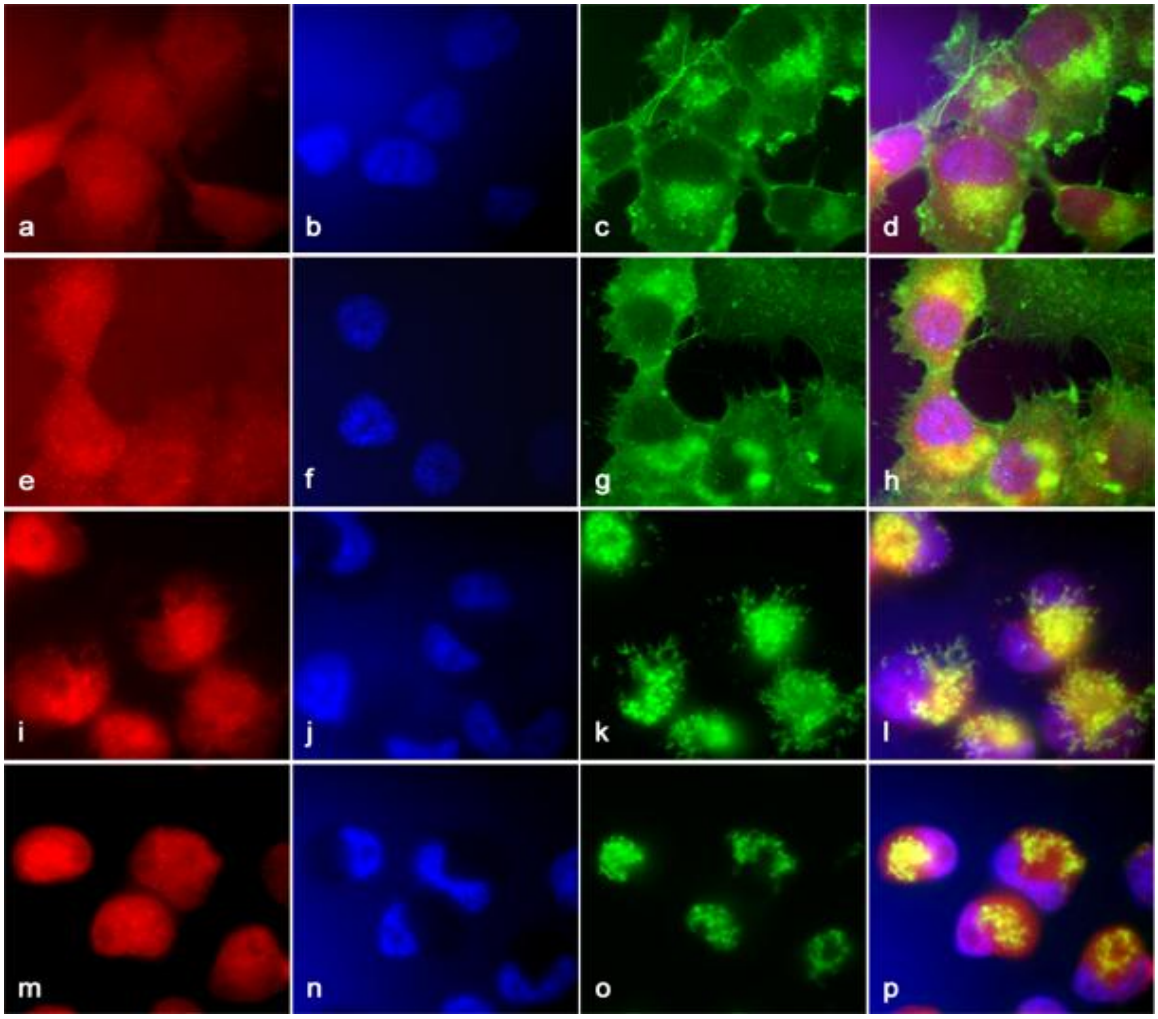


Figure 7: H1299 cells stained with phospho-p38 MAPK (red), actin (green), and nuclei (blue). Cells were grown on coverslips then treated with Cuc IIa for 2 days. Cuc IIa treatments: 0µg/mL (a-d), 1µg/mL (e-h), 10µg/mL (i-l), and 50µg/mL (m-p). phospho-p38 MAPK (a, e, i and m), nuclei (b, f, j and n), actin (c, g, k and o), and merge (d, h, l, and p).

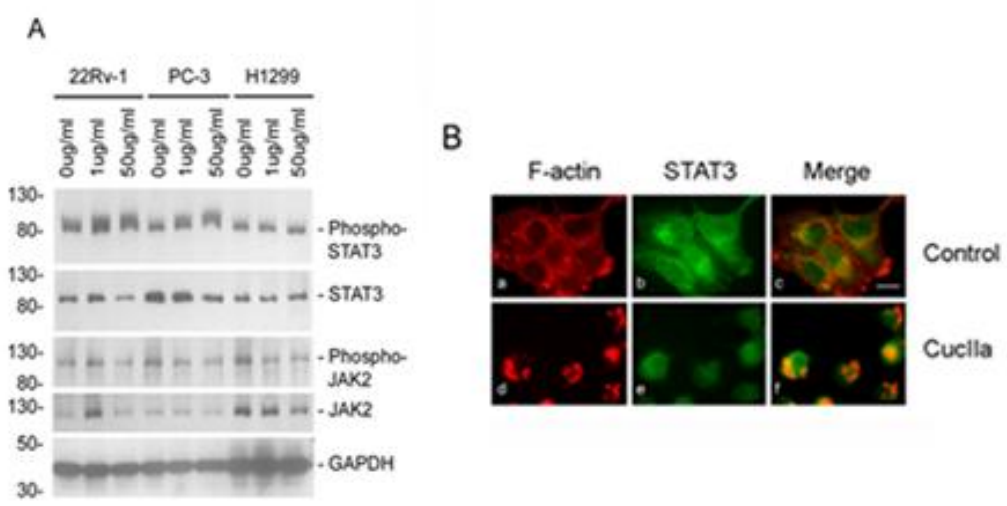


Figure 8: A. Western blots of JAK2 and STAT3 proteins alongside phosphorylated counterparts. GAPDH is used as control for equal loading of lysate proteins. B. Immunofluorescence staining of actin (red; a,d), STAT3 (green; b, e), and merge (c, f).

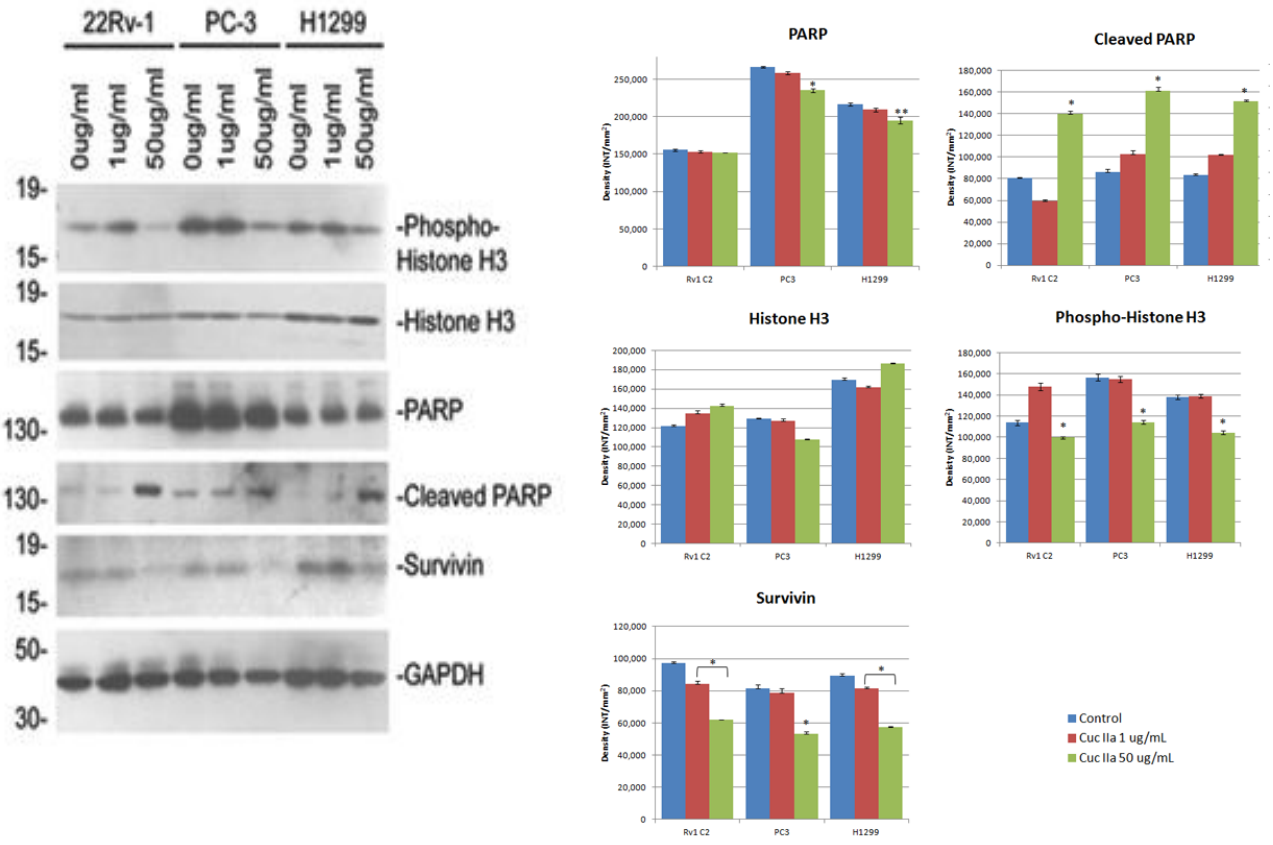
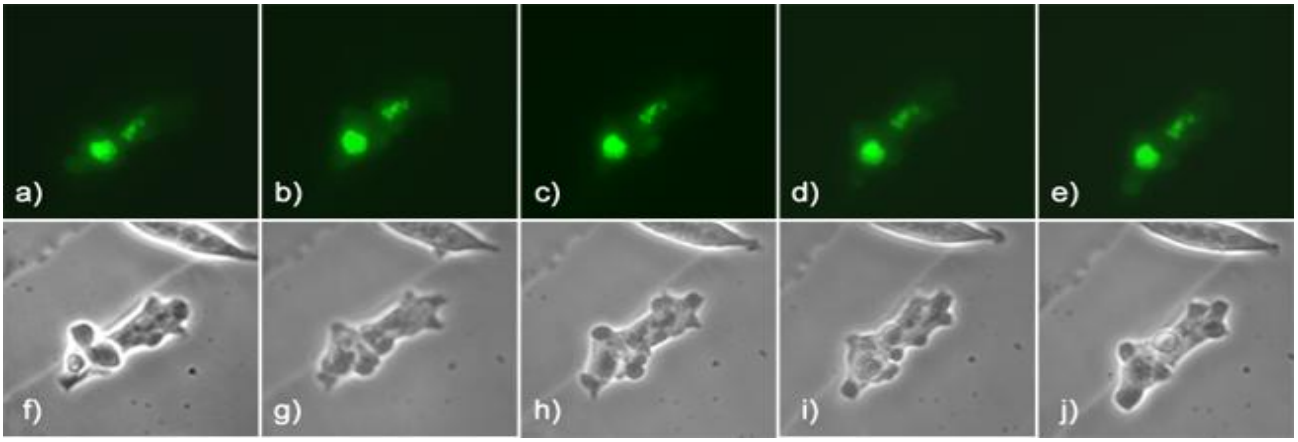


Figure 9: (left) 22Rv-1, PC3 and H1299 cells were treated with 1 µg/mL and 50 µg/mL Cuc IIa, respectively. Resulting lysates were probed by western blots for cleaved and total PARP, Survivin, phospho- and Histone H3 with GAPDH to ensure equal loading of protein.

(right) Densitometry analysis performed on Survivin, phospho-Histone H3, Histone H3, cleaved PARP, and PARP. * $p<0.01$, ** $p<0.05$.

Panel A



Panel B

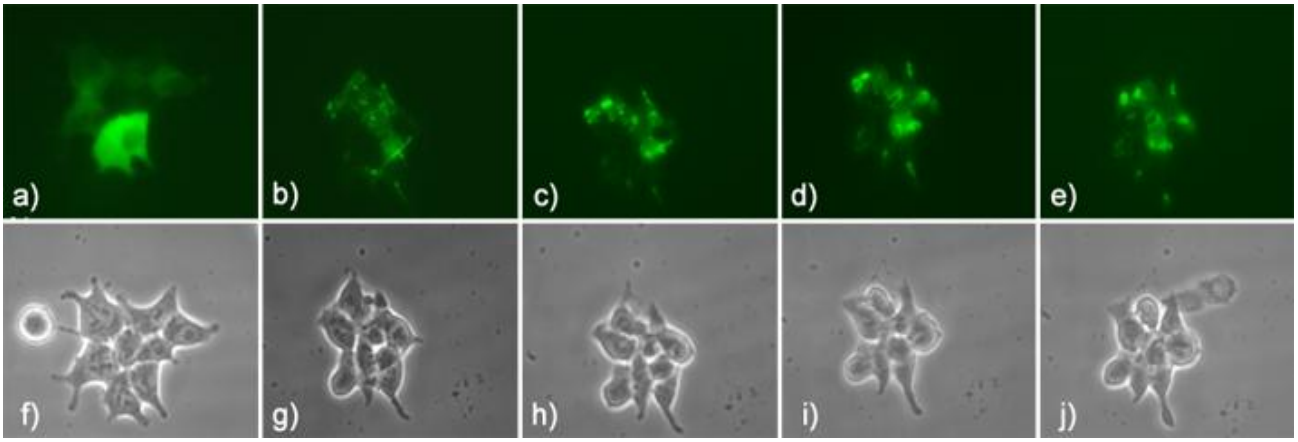


Figure 10: CWR22Rv-1 cells were transfected with EGFP-actin and observed over a period of 4 hours using an inverted fluorescent microscope. Pictures were recorded every 15 minutes after which images were compiled into a single figure for comparison. Over the period of observation, there is a clear difference between the actin of non-treated cells as compared to treated cells. Non-treated cells maintained a normal distribution of actin throughout the cell, while Cuc IIa treated cells showed an increased clustering of actin as the cells were exposed to treatment. Panel A: Non-treated Cuc IIa, Panel B: Cuc IIa 10µgml.

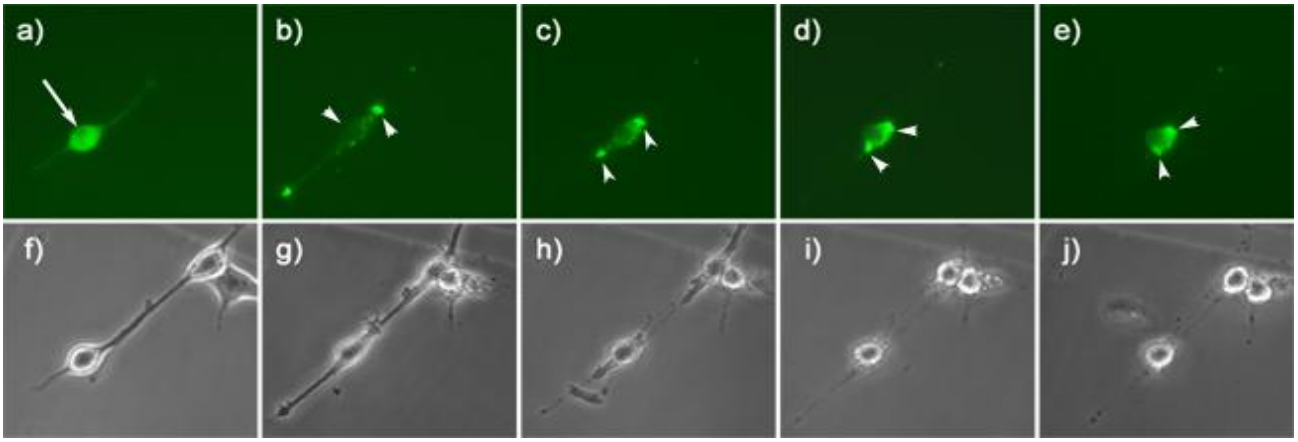


Figure 11: NIH 3T3 cells transfected with EGFP-actin, treated with Cuc IIa at 10 μg/mL and observed over a period of 4 hours on inverted fluorescent microscope where images were taken every 15 minutes. The images were compiled into a single figure for comparison of cells treated over time. As with CWR22Rv-1 cells, we showed actin clusters of NIH 3T3 cells treated with Cuc IIa at 10μg/mL.

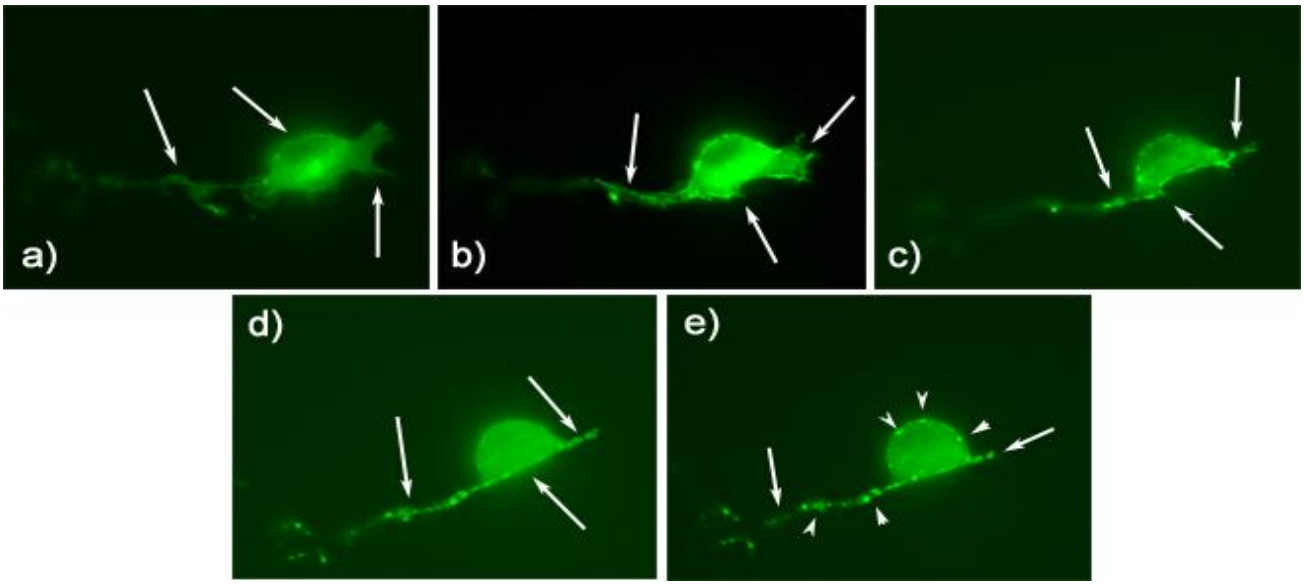


Figure 12: NIH 3T3 cells were transfected with EGFP-Actin, set up under inverted fluorescent microscope to record images every 15 minutes for 4 hours and treated with Cuc IIa at 50 μg/mL. As we expected, the actin clusters were detected as early as 1 hour (b). The treatment was rinsed off using normal medium after 2 hours (c) exposure of Cuc IIa. The cells were continued to be monitored for any indication that the normal actin morphology could be recovered from the treatment. The images taken at hours 3 (d) and 4 (e) showed that cells are unable to be recovered after treatment with Cuc IIa.

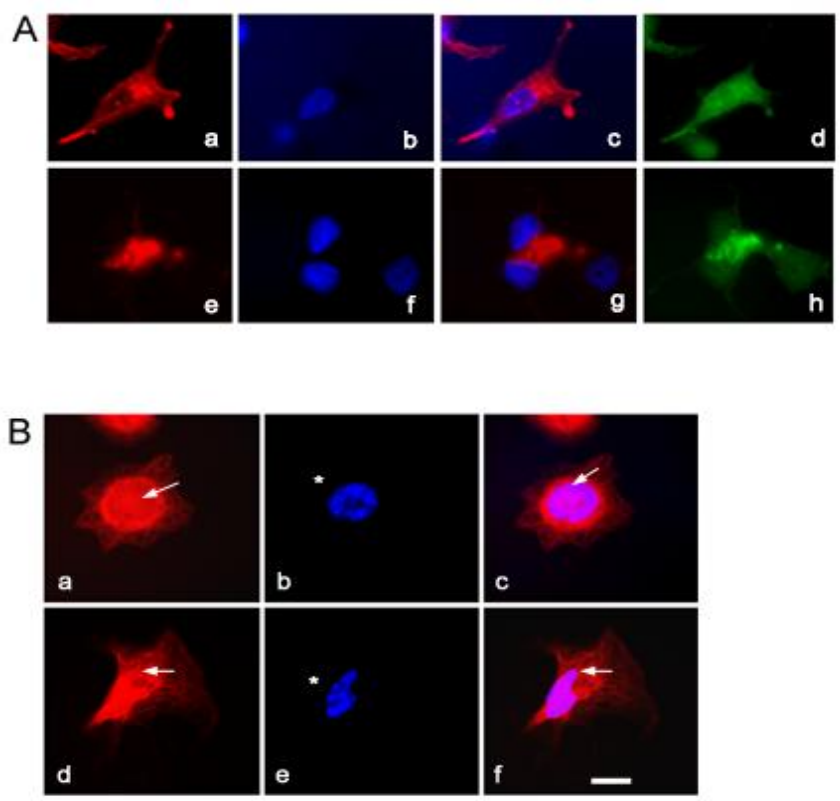


Figure 13: CWR22Rv-1 cells were grown in culture medium and treated with 50 µg/mL Cuc IIa for 24 hours. Cells were stained for A) actin (red) and B) tubulin (red). A) Actin staining for EGFP transfected cells; a-d non-treated cells; e-h Cuc IIa 50µg/mL. B) Tubulin staining; a-c non-treated cells, d-f 50µg/mL Cuc IIa.

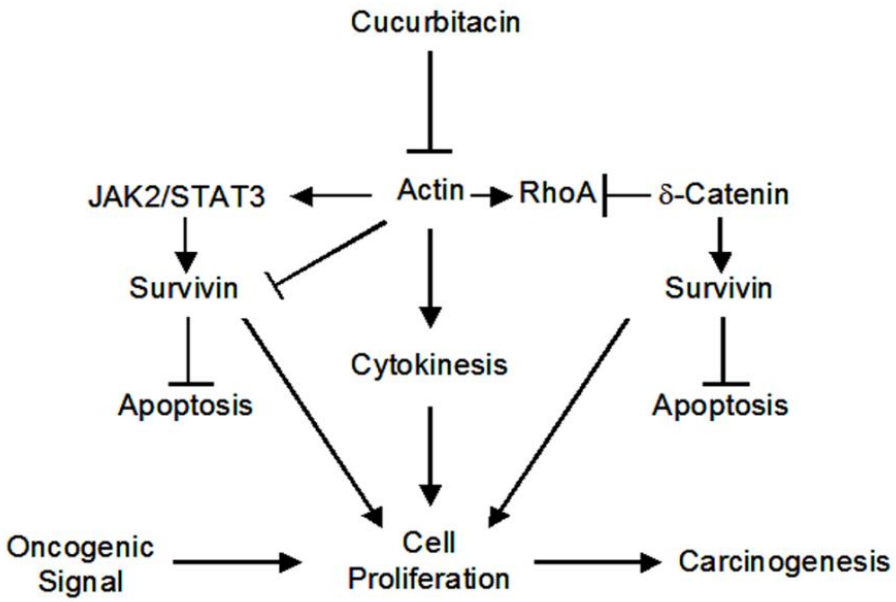


Figure 14: Schematic model illustrating the potential mechanism of Cuc IIa-induced apoptosis

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